Title: T-2 Toxin-3α-glucoside in Broiler Chickens : Toxicokinetics, Absolute Oral Bioavailability, and in Vivo Hydrolysis

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Keywords

Modified – Masked – Mycotoxin – Toxicokinetics

1. Introduction

Fusarium is regarded as one of the most important mycotoxigenic fungi genera. It is known to produce several classes of mycotoxins of which the trichothecenes are the most important based on occurrence. From this class of mycotoxins, type A trichothecenes are the more toxic subclass. T-2 toxin (T-2)is the most acute toxic mycotoxin of the type A trichothecenes. It is commonly found in cereals and several European surveys have reported T-2 incidences over 80% for certain cereals and maximum concentrations up to 2,406 µg/kg have been observed in naturally contaminated oats [1-4].

Poultry is considered to have a high exposure to T-2 as their diet is mainly cereal based. Toxic effects in poultry include inhibition of protein, DNA and RNA synthesis, immunomodulation, gastro-intestinal cell lesions, organs and neural disturbances [5]. At subclinical levels decreased performance parameters can be observed [6].

For broiler chickens, first adverse effects like mucosal damage in the oral cavity occur at a dose of 40 µg/kg body weight (bw) per day. For laying hens, a reduction of the egg production and hatchability were observed at doses of 120 µg/kg bw per day [6]. Based on these data the European Union has issued indicative levels for the sum of T-2 and HT-2 toxin (HT-2) in food and feed commodities, from above which investigations should be performed, certainly in case of repetitive findings [7, 8]. For poultry compound feed, the indicative level is set at 250 µg T-2 + HT-2/kg feed.

The indicative levels suggested by legislative authorities only take into account free mycotoxins, such as T-2 and HT-2. However, based on recent research this appears to be an incomplete approach, as additionally to free mycotoxins, food and feed is often co-contaminated with modified mycotoxins. For T-2, *in planta* glycosylation of the 3-hydroxyl group to T-2 toxin-β-glucoside (T2-G) could be an important contributor to modified forms

based on recent studies. The occurrence of T2-G in naturally infected wheat and oats has been described by Lattanzio *et al*. (2011). Based on High-Resolution Mass Spectrometry (HRMS) peak area ratios between glucoside derivatives and free T2 and HT-2 in naturally contaminated wheat and oats samples, amounts of glycosylated T-2 and HT-2 as high as 27% of their deglycosylated counterparts were demonstrated [9]. Furthermore, Busman *et al*. and Lattanzio *et al*. observed that glycosylation is not only part of a detoxification strategy by plants, but can also be a relevant route of fungal metabolism [10, 11].

The European Food Safety Authority (EFSA) recently published a scientific opinion on modified mycotoxins. In this opinion the relative contribution of modified forms of T-2 to the occurrence of T-2 was calculated to be 10%. The risk of this T2-G contribution was assessed for humans and several animal species (pigs, ruminants, horses, poultry and dogs) where possible. The combined risk of T-2, HT-2 and their modified forms was found not to be of concern, except for the highest upperbound exposure for toddlers, with levels slightly exceeding the TDI. Furthermore, the risk assessment assumed that all modified forms have the same toxicity as their parent compounds, and this due to the lack of information on bioavailability and toxicity of these modified forms [1]. However, as shown by several studies, modified mycotoxins may have a significantly altered inert toxicity and oral bioavailability; in addition they may be subject to *in vivo* hydrolysis as well [12-14]. These knowledge gaps pose an additional uncertainty on the derived conclusions of this scientific opinion.

Presently, no toxicokinetic data is available for T2-G in humans nor animals. Determination of the absolute oral bioavailability, *in vivo* hydrolysis and toxicokinetic characteristics could significantly contribute to an accurate risk assessment by legislative authorities on modified mycotoxins such as T2-G.

2. Experimental

2.1 Compounds, standards and solutions

T-2, HT-2 and T-2 triol were purchased from Fermentek (Jerusalem, Israel). T2-G was synthesized by the United States Department of Agriculture (USDA) as described [15]. All compounds were dissolved in ethanol yielding a stock solution of 10 mg/mL. The internal standard (IS), 25 µg/mL $^{13}C_{24}$-T-2 toxin solution in acetonitrile, was purchased from Sigma Aldrich (Bornem, Belgium). All stock solutions were stored at ≤−15 °C. Individual working

standard solutions of 100 µg/mL were prepared by diluting the above stock solutions with HPLC-grade acetonitrile. All standard solutions were stored at ≤−15 °C. Standard mixture working solutions (containing T-2, HT-2, T-2 triol and T2-G) of 5,000, 500, 50 and 5 ng/mL were prepared by mixing and diluting the individual working standard solutions in HPLC-grade acetonitrile and were kept at 2-8 °C. Acetonitrile solutions of T-2 and HT-2 have been reported stable for 24 months at 25 °C [16]. For T-2 triol no published stability data is available. However, suppliers claim a stock solution shelf-life of at least one month at ≤−15 °C. Regarding synthesised T2-G, no data is published on storage and stability. During the study no degradation of the T2-G solutions into T-2, HT-2 and T-2 triol could be detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.2 Instrumentation, materials & reagents

Separation of analytes was achieved on an Acquity UPLC® BEH C18 column (150 x 2.1 mm i.d., 1.7 µm) with an Acquity UPLC® BEH C18 column VanGuard pre-column (5 x 2.1 mm i.d., 1.7 µm). An Acquity UPLC system coupled to a Xevo® TQ-S mass spectrometer was used, operating in positive electrospray ionization (ESI^+) mode, all from Waters (Zellik, Belgium). Microfilters Durapore PVDF (0.22 µm) were obtained from Millipore (Overijse, Belgium). Water, methanol, acetic acid and ammonium acetate (all UHPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (HPLC grade) was obtained from VWR (Leuven, Belgium).

2.3 Plasma sample preparation

Blank plasma samples. Heparinized blood from 12 h fasted broiler chickens (Ross 308, Institute for Agricultural and Fisheries Research (ILVO) animal unit, Merelbeke, Belgium) was centrifuged (2,851 x *g*, 10 min, 4 °C) to obtain blank plasma. This plasma was pooled, homogenized and stored in 15 mL aliquots at ≤ – 15 °C until the moment of use as matrix-matched calibrators and quality control samples.

Calibrator and quality control samples. To 200 µL of blank plasma, 20 µL of a 100 ng/mL IS working solution and appropriate volumes of the standard mixture working solutions (5,000, 500, 50 and 5 ng/mL) were added to obtain calibrator samples with mycotoxin concentrations of 0.1, 1, 5, 10, 50, 100, 500 and 1,000 ng/mL. Acetonitrile was added up to a volume of 1.0 mL to precipitate plasma proteins. After vortex mixing and

centrifugation (8,517 x *g*, 10 min, 4 °C), the supernatant was isolated and evaporated to dryness under nitrogen at 40 °C. The sample was then redissolved in 200 µL of a UHPLC-grade water/methanol mixture (85/15; v/v), filtered (0.22 µm) and analyzed by LC-MS/MS.

Incurred samples. To 200 µL of plasma, 20 µL of a 100 ng/mL IS working solution were added and the samples were subjected to the same sample preparation procedure as the calibrator samples.

2.4 LC-MS/MS analysis

Analytes were eluted at a flow rate of 300 µL/min with mobile phase A consisting of water, methanol and acetic acid (97/2/1; v/v/v) + 5 mM aqueous ammonium acetate, and mobile phase B consisting of water, methanol and acetic acid (2/97/1; v/v/v) + 5 mM ammonium acetate. A gradient elution program was applied: 0-1 min (70% A, 30% B), 1.0-1.1 min (linear gradient to 50% B), 1.1-5.0 min (50% A, 50% B), 5.0-5.1 min (linear gradient to 95% B), 5.1-8.0 min (5% A, 95% B), 8.0-8.1 min (linear gradient 30% B), 8.1-10.0 min (70% A, 30% B). Column and autosampler temperatures were set to respectively 40 and 8 °C, injection volume (partial loop) was fixed at 10 µL.

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with two ion transitions for each target analyte. Instrumental and compound specific parameters were optimized by the direct infusion of 10 ng/mL standard solutions in a 50/50 (v/v) mixture of mobile phase A and B at a flow rate of 50 µL/min. Capillary voltage was set at 2.5 kV, cone at 15 V, cone gas flow 150 L/h, desolvation temperature at 600 °C, desolvation gas flow at 1000 L/h and collision gas flow at 150 µL/min. All compounds were detected in ESI^+. Compound specific instrumental parameters, together with precursor and product ions used for quantification and qualification, are given in **Table 1**.

2.5 Validation

Validation was performed on spiked blank plasma samples. As validation guidelines recommendations by the European Community [17, 18] and the Veterinary International Conference on Harmonisation (VICH) [19] were utilized.

Linearity of the response of the compounds was assessed by means of three matrix-matched calibration curves consisting of at least seven calibration points in the range of 0.1-100 ng/mL for HT-2, T-2 triol and T2-G and in the range of 0.1-1,000 ng/mL for T-2. Utilised

criteria and corresponding acceptability ranges were correlation coefficients (*r*) ≥0.99 and goodness-of-fit coefficients (gof) ≤20%.

Within-day accuracy & precision were determined by analyzing six samples at a low concentration level (limit of quantitation or LOQ of the analytes) and at a high concentration level (range maximum). Values for the relative standard deviation (RSD) should not exceed 2/3 of the $RSD_{max,}$ calculated according to the Horwitz equation [20]. The acceptance criteria for accuracy were: −50% to +20%, −30% to +10% and −20% to +10% for concentrations ≤ 0.1 ng/mL, between 1 and 10 ng/mL, and ≥10 ng/mL, respectively.

Between-day accuracy & precision were assessed by analyzing the LOQ and range maximum in threefold on three consecutive days (n=3x3). The acceptance criteria for accuracy were identical to the values given above and RSD values should not exceed the RSD_{max} [20].

The *LOQ* was calculated as the lowest concentration for which the method had acceptable results with regards to accuracy and precision. It was determined by spiking 6 plasma samples at 0.1, 0.2, 0.5, 1 or 2 ng/mL. The LOQ was also established as the lowest point of the calibration curve. The *limit of detection* (*LOD)* was calculated using the signal-to-noise (S/N) ratio of the samples spiked at the LOQ level (n=6) corresponding to the concentration with a S/N ratio of 3.

Carry-over was evaluated by analysing a mixture of mobile phase A and B (50/50; v/v) directly after the highest calibrator.

The *specificity*, the capability of the method to distinguish signals of the analytes from other substances or interferences, was determined on six blank plasma samples. For an acceptable specificity the S/N ratio of possible interfering peaks with similar retention times in these samples could not exceed the S/N ratio of the analyte(s)' LOD.

Recovery and matrix effects. Two types of matrix-matched calibration curves were prepared, one by spiking the blank calibrator samples before and one after extraction. A third calibration curve was prepared in standard solution. All curves consisted of five calibration points in the range of 1-100 ng/mL. The slopes of these calibration curves were compared to calculate the apparent recovery (R_A = 100 × slope spiked before extraction/slope standard solution), the matrix effect denoted as signal suppression/enhancement (SSE = 100 × slope spiked after extraction/slope standard solution) and the recovery of the extraction step (R_E = 100 × slope spiked before

extraction/slope spiked after extraction). Regarding SSE, values ≤ 100% indicate ion suppression due to matrix effect, values ≥ 100% are caused by ion enhancement [21].

2.6 Animal trial

Six broilers (Ross 308, mixed sex, 18 days of age) were housed at the Faculty of Veterinary Medicine of Ghent University. Water and feed were present *ad libitum*. The animals were allowed to acclimatize for 1 week in a climate-controlled stable with temperatures between 18 and 25 °C, a relative humidity between 40 and 80% and a 18 h/6 h light/dark cycle. Three broilers were given a single T2-G intravenous injection (IV) and three broilers a single per os bolus (PO, by means of gavage directly into the crop). After a one day washout period, the animals were administered T-2 by IV or PO in a two-way cross-over design with a one day washout period (**Figure 1**). Twelve hours before administration of T-2 or T2-G the animals were fasted. In order to obtain detectable plasma concentrations after PO administration it was opted to use 1 mg/kg T2-G and an equimolar dose of T-2, 0.74 mg/kg. Mycotoxin solutions (10 mg/mL in ethanol) were diluted with saline (0.9% NaCl, VWR, Leuven, Belgium) to an administration volume of one mL. Before the administration of the toxins (0) and 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min afterwards, blood (0.5 – 1 mL) was collected. The animal trial was approved by the ethical committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering (EC2013/139).

2.7 Toxicokinetic modeling and statistical analysis

Toxicokinetic analysis was performed using WinNonlin Professional version 5.2.1. (Pharsight, St-Louis, MO, USA). Plasma concentrations below the LOQ were not taken into account. For the analysis of T-2 and T2-G, a two-compartmental model (central compartment (Vc) and peripheral compartment (Vp)) with first-order elimination was applied.

The following primary and secondary toxicokinetic parameters were calculated from the IV data: total body clearance (CL), central volume of distribution (Vc), peripheral volume of distribution (Vp), disposition rate constant (β) and disposition half-life (t1/2β). Absorption rate constant (ka), maximal plasma concentration (C_{max}) and time to maximal plasma concentration (t_{max}) were calculated based on the PO data. A $1/\hat{y}$ weighing was applied for all calculations.

F, determined for T-2 and T2-G, indicates the absolute oral bioavailability, *i.e*. the fraction absorbed in the systemic circulation in its unchanged form.

$$F\ T2G = \frac{AUC_{\text{T2G 0–inf after PO T2G}}}{AUC_{\text{T2G 0–inf after IV T2G}}}$$

FRAC, determined for T-2 and T2-G, is the absorbed fraction of the dose in either form, calculated by correcting for molecular weight (MW) and CL.

$$FRAC\ T2G = \frac{\frac{AUC_{\text{T2G 0–inf after PO T2G}} * \text{CL}_{\text{T2G}}}{MW_{T2G}} + \frac{AUC_{\text{T2 0–inf after PO T2G}} * \text{CL}_{\text{T2}}}{MW_{T2}}}{\frac{AUC_{\text{T2G 0–inf after IV T2G}} * \text{CL}_{\text{T2G}}}{MW_{T2G}}}$$

Presystemic hydrolysis (Pres. Hydr.) of T2-G is the percentage of the total dose that is hydrolysed presystemically to T-2 and absorbed as T-2 (%), calculated by correcting for molecular weight (MW) and CL.

$$Pres.Hydr. = \frac{\frac{AUC_{\text{T2 0–inf after PO T2G}} * \text{CL}_{\text{T2}}}{MW_{T2}}}{\frac{AUC_{\text{T2G 0–inf after PO T2G}} * \text{CL}_{\text{T2G}}}{MW_{T2G}} + \frac{AUC_{\text{T2 0–inf after PO T2G}} * \text{CL}_{\text{T2}}}{MW_{T2}}}$$

Statistical analysis of FRAC, F, C_{max} and t_{max} after T2-G administration compared to after T-2 administration consisted of one-way ANOVA with post-hoc Scheffé tests ($p < 0.05$) (SPSS 20.0, IBM, Chicago, IL). All variables were log transformed in order to fulfill the equality of variances criterion as determined by the Levene's test for homogeneity of variances (p value > 0.01).

3. Results and discussion

The results for linearity, r and gof, sensitivity, LOD and LOQ, are given in **Table 2**. The results for linearity were all in accordance with the acceptance criteria, with $r \geq 0.99$ and gof

≤ 20%. LOD varied from 0.01 (T-2 triol), 0.02 (T-2 and T2-G) to 0.04 (HT-2) ng/mL. LOQ was 0.1 ng/mL for all mycotoxins. **Table 3** displays the results for accuracy (%) and precision (RSD, %). All results fell within the specified acceptability ranges.

The method was specific as no interfering peaks were detected in the chromatographic elution zone of the analytes with S/N values ≥ 3 (results not shown). Furthermore, carry-over was evaluated (results not shown). For none of the compounds signals were detected that could interfere with the response/area of the analytes at their given retention time.

In preliminary studies administered doses calculated based on EU maximum guidance values in feed could not be detected in plasma of broilers after PO administration [22]. Consequently, this set up did not allow to calculate the absolute oral bioavailability of T-2. A recent study by Sun et al. orally dosed broilers at 2.0 mg T-2 per kg bw, resulting in detectable plasma profiles [23]. Consequently, the initially planned dose was increased to use 1 mg T2-G/kg bw and an equimolar doses of T-2, 0.74 mg/kg bw.

Plasma concentration-time profiles for PO and IV administration of T-2 (n=6) and T2-G (n=3) to broiler chickens are presented in **Figure 2**. Each profile is represented as the mean ± standard deviation (SD).

During the animal trial none of the broiler chickens showed any adverse effects after acute IV or PO administration of T-2G. Within 24 h after the T-2 cross-over one broiler died. However, this could not be directly related to the administration of the mycotoxin. The other broilers showed no clinical symptoms of the T-2 IV and PO administration.

A first observation from the plasma concentration-time profiles is that after PO and IV administration of T-2 and T2-G to broilers no T-2 triol and only traces of HT-2 were detected in plasma. In contrast, Sun *et al*. detected high amounts of HT-2 and T-2 triol after IV administration of T-2 (0.5 mg/kg bw), as well as high concentrations of T-2 triol after PO T-2 administration (2 mg/kg) [23].

The calculated F of T-2 in chickens is 2.17±1.80%. Sun *et al*. calculated a notably higher F of T-2, i.e. 17.1% [23]. Osselaere *et al*. administered T-2 IV and PO (15 µg/kg bw) to chickens and no plasma levels above the LOQ could be observed for T-2 and its metabolite HT-2. Consequently, no determination of F was possible. Given the LOQ of 2.5 ng/mL of the latter method, it does indicate a likely low F, in correspondence with the findings of this study.

The values for CL (191 mL/min/kg), β (0.0346/min), t1/2β (21.4 min) and Vss (sum of Vc and Vp; 3240 mL/kg) correspond well with the values obtained by Sun *et al*., i.e. 120 mL/min/kg, 0.04/min, 17.3 min and 3170 mL/kg, respectively [23]. In contrast, the values for these toxicokinetics parameters described by Osselaere *et al*. differ significantly, this discrepancy is probably caused by the application of a non-compartmental model [22].

For T2-G a FRAC and F of 10.4±8.7% and 10.1±8.5% were determined, respectively. This slight difference is caused by a partial presystemic hydrolysis, 3.85±1.31%, of T2-G to T-2. The FRAC of T2-G is five times higher than that of T-2. Mean T2-G volumes of distribution, CL and C_{max} values were significantly larger than those observed for T-2. Mean values for β (0.0285/min) and t1/2β (24.3 min) of T2-G do not significantly differ from those obtained for T-2.

These differences in toxicokinetics parameters between T-2 and T2-G, specifically the five times higher FRAC for T2-G, clearly indicate the flaw in assuming equal toxicity of modified and parent compounds in risk assessment [1]. For every amount of T2-G in feed, compared to T-2, the systemic exposure to the glucoside conjugate will be five times higher. Consequently, the contribution in feed, estimated at 10% would result in an *in vivo* contribution of 50%. This clearly indicates the flaw in assuming equal toxicity of modified and parent compounds in risk assessment.

Assuming equal intrinsic toxicity is similarly flawed. Although no T2-G toxicity data is available, a clearly decreased toxicity has been demonstrated for deoxynivalenol-3-glucoside (DON3G) compared to DON [21,24-26]. Given the high degree of structural similarity between DON and T-2, a similar decrease in toxicity may reasonably be expected for T2-G. Furthermore, as seen with DON3G, T2-G was not hydrolysed in broiler chickens to its respective free mycotoxin in a significant extent. Consequently, although a relatively higher oral bioavailability and absorbed fraction were observed, further research is needed as the contribution of these modified forms to the overall toxicity of these mycotoxins may be limited due to a probable low intrinsic toxicity as well as absence of a significant degree of hydrolysis.

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Figure 1. Two-way cross-over design (IV and PO) for T-2 (blue) in six broilers with a one day washout period. T2-G (green) was administered IV and PO to each 3 broilers.

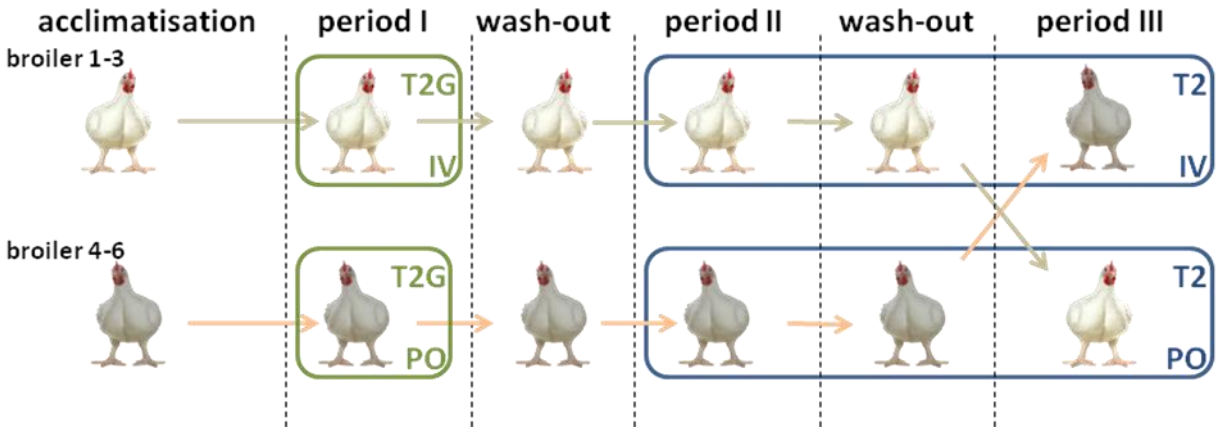

Figure 2. Plasma concentration-time profiles of T-2 toxin (T-2) and T-2-glucoside (T2-G) after oral (PO) and intravenous (IV) administration of T-2 (0.76 mg/kg bodyweight, n=6) and T2-G (1 mg/kg bodyweight, n=3) to broiler chickens. Values are presented as mean + SD.

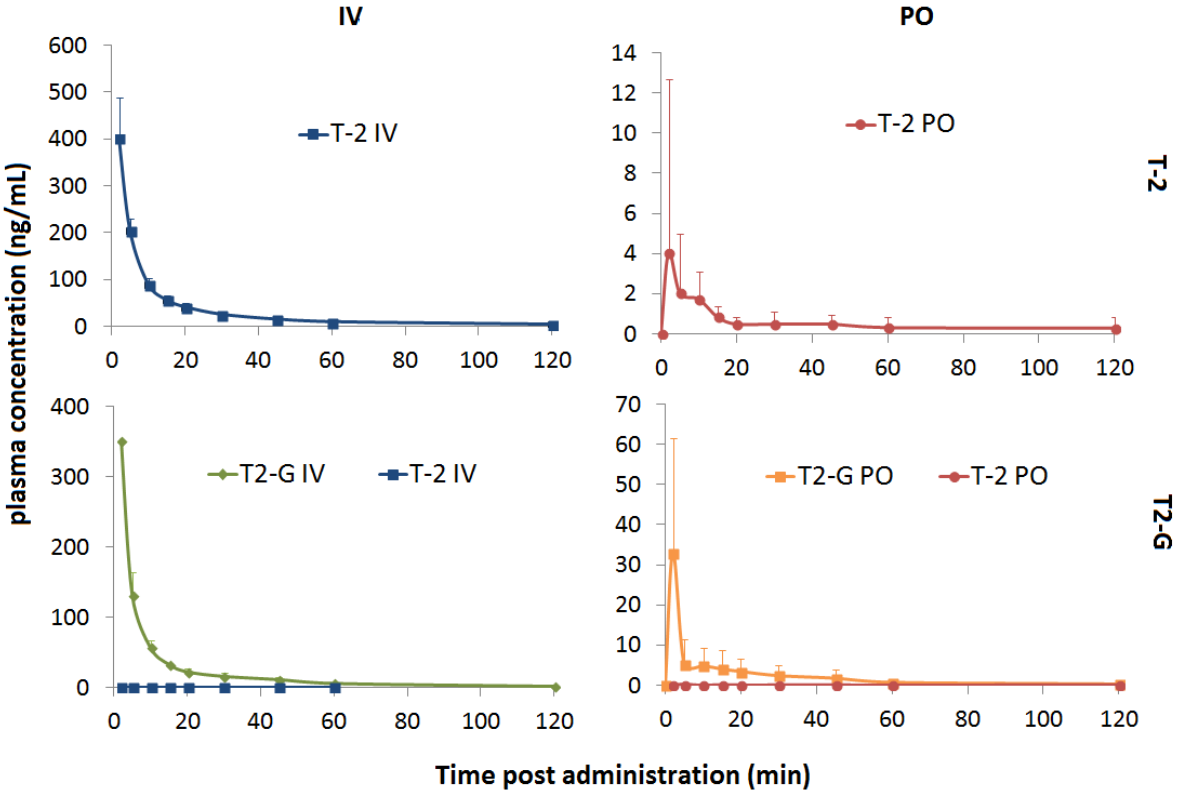

Table 1. Compound specific MRM ion transitions and MS-parameters; Rt= retention time; IS= internal standard; [a] quantifier ion

	Measured form/adduct	Precursor ion (m/z)	Product ion (m/z)	Rt (min)	Collision Energy
T-2	$[M+NH_4]^+$	484.1	245.4[a] 305.4	5.7	12 12
HT-2	$[M+Na]^+$	447.2	285.1[a] 345.1	4.2	20 18
T-2 triol	$[M+Na]^+$	405.0	124.9[a] 303.0	3.3	15 15
T2-G	$[M+Na]^+$	650.9	326.9[a] 406.9	4.5	40 40
$^{13}C_{24}$-T-2 (IS)	$[M+NH_4]^+$	508.2	198.0[a] 228.9	5.7	22 22

Table 2. Validation results for linearity (r & gof, minimum 7 concentration points), sensitivity (LOD & LOQ; n=6), matrix effects (SSE), apparent recovery (R_A) and extraction recovery (R_E)

	Range (ng/mL)	Correlation coefficient (r)	Goodness of fit (gof)	LOD (ng/mL)	LOQ (ng/mL)	SSE (%)	R_A (%)	R_E (%)
T-2	0.1-1000	0.9999	6.52%	0.02	0.1	96.6	77.8	80.5
HT-2	0.1-100	0.9981	17.33%	0.04	0.1	31.6	81.3	257
T-2 triol	0.1-100	0.9984	10.79%	0.01	0.1	19.7	55.4	282
T2-G	0.1-100	0.9910	13.36%	0.02	0.1	41.8	76.7	183

Table 3. Validation results for within-day precision (n=6) and between-day precision (n=3x3) with corresponding accuracy at low (LOQ) and high (Linearity range maximum) concentration level

	Within-day (n=6)				Between-day (n=3x3)			
	Accuracy (%)		Precision (RSD, %)		Accuracy (%)		Precision (RSD, %)	
	LOQ	Range max	LOQ	Range max	LOQ	Range max	LOQ	Range max
T-2	8.3	-0.4	18.8	2.6	11.1	-0.5	7.0	1.4
HT-2	-42.0	-6.0	18.9	2.7	-40.0	-5.1	18.6	2.7
T-2 triol	-10.0	-4.5	17.6	2.1	-1.1	-3.3	19.2	2.8
T2-G	-9.-9	-3.8	19.6	2.3	-31.1	-3.2	30.2	2.5

Table 4. Toxicokinetic parameters, absolute oral bioavailability (%) and presystemic hydrolysis (%) of T-2 (n=6) and T2-G (n=3) after IV to broiler chickens.

	T-2	T2-G
β (1/min)	0.0346±0.0088	0.0285±0.0017
t1/2β (min)	21.4±6.6	24.3±1.4
CL (mL/min/kg)	191±42	377±85*
Vc (mL/kg)	1173±387	2705±1281*
Vp (mL/kg)	2067±744	4568±1838*
FRAC (%)	2.17±1.80	10.4±8.7*
Pres.Hydr. (%)	-	3.49±1.19*
F (%)	2.17±1.80	10.1±8.5*
C_{max} (ng/mL)	5.55±7.49	33.9±27.0*
t_{max} (min)	8.14±4.38	4.67±4.62

Values are presented as mean ± SD. ke=elimination rate constant (1/min); t1/2el=elimination half-live (min); CL=clearance (mL/min/kg); Vc=central volume of distribution (mL/kg); Vp=peripheral volume of distribution (mL/kg); FRAC=absorbed fraction (%);

Pres.Hydr.=percentage of the total dose that is hydrolysed presystemically (%); F=absolute oral bioavailability (%); C_{max}=maximum plasma concentration (ng/mL); t_{max}= time to maximum plasma concentration (min); Statistical analysis was performed by means of ANOVA,* statistically significant difference ($p<0.05$)